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Epstein-Barr Virus Latent Membrane Protein-1 Effects on Junctional Plakoglobin and Induction of a Cadherin Switch

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Abstract

Latent membrane protein-1 (LMP1) is considered the major oncoprotein of Epstein-Barr virus (EBV) and is frequently expressed in nasopharyngeal carcinoma (NPC). LMP1 promotes growth and migration of epithelial cells, and the loss of plakoglobin has been identified as a contributing factor to LMP1-induced migration. Plakoglobin is a junctional protein that can also serve as a transcription factor in Tcf/Lef signaling. To determine the effects of LMP1 on the molecular and functional properties of plakoglobin, LMP1 was over-expressed in the NPC cell line, C666-1. LMP1 did not affect plakoglobin stability but did decrease plakoglobin transcription. The resultant decreased levels of nuclear plakoglobin did not affect Tcf/Lef activity or the amount of plakoglobin bound to Tcf4. Although LMP1 induced and stabilized β-catenin, a protein with common binding partners to plakoglobin, the loss of plakoglobin did not affect its association with Tcf4. However LMP1 did induce a cadherin switch from E- to N-cadherin, a process involved in cancer progression, and enhanced the association of junctional β-catenin with N-cadherin. LMP1 decreased overall levels of junctional plakoglobin but the remaining junctional plakoglobin was found associated with the induced N-cadherin. This increased association of junctional plakoglobin with N-cadherin was a distinguishing feature of LMP1-expressing cells that have reduced migration due to restoration of plakoglobin. Low levels of plakoglobin were also detected in human NPC tissues. These findings reveal that the effects of LMP1 on junctional plakoglobin and the initiation of a cadherin switch likely contribute to metastasis of NPC.

Keywords

Epstein-Barr virus; latent membrane protein-1; plakoglobin; β-catenin

Introduction

Epstein-Barr virus (EBV) is a ubiquitous γ herpesvirus that is associated with malignancies of epithelial and lymphoid origins. These EBV-associated malignancies include Burkitt lymphoma, nasopharyngeal carcinoma (NPC), Hodgkin disease and the development of lymphomas in immunosuppressed patients (1). The transforming potential of EBV has been linked to the expression of its latent genes including latent membrane protein 1 (LMP1), EBNA-1, EBNA-2, EBNA-3A and EBNA-3C, which are required for EBV-induced B cell transformation (2). The expression of LMP1 and LMP2A is frequently detected in NPC and

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likely contribute to the development of cancer (1). LMP1 is considered the major oncoprotein of EBV such that expression of LMP1 alone can transform Rat-1 and human embryonic lung fibroblasts inducing focus formation, anchorage independent growth in soft agar, and tumor formation in nude mice (3,4). Additionally, *in vivo* LMP1 transgenic mice develop B cell lymphomas and epidermal hyperplasia (5,6). LMP2A also has oncogenic potential and is able to inhibit keratinocyte differentiation to promote the transformation of several epithelial cell lines (7–10).

LMP1 activates multiple signaling pathways that regulate growth and migration (11–20). In epithelial cell transformation, these include the activation of transcription factors (NF-κB, AP1, Id and Stats) (12–16), proteins that modulate adhesion and invasion (E-cadherin, MMP9 and MUC1) (17,18), and signaling pathways (PI3K/Akt, ERK, p38 and JNK) (3,11,14). The growth of B lymphocytes from LMP1 transgenic mice requires the activation of Akt, NFκB and Stat3 signaling (19), of which Akt and NFκB pathways are also required for LMP1-induced transformation of the EBV-positive NPC cell line, C666-1 cells (20). Expression of LMP1 in C666-1 cells also induces growth in soft agar, enhances migration and decreases plakoglobin expression (20). Plakoglobin, also known as γ-catenin, is a junctional protein found at adherens junctions and desmosomes. It is a member of the armadillo protein family and is highly homologous to but is not functionally interchangeable with β-catenin (21,22). Like β-catenin, plakoglobin also associates and regulates the Tcf/Lef transcription factors (22). The effects on plakoglobin required both of the major signaling domains of LMP1, COOH-terminal activation regions (CTAR) 1 and 2 (20). LMP1 also affects other junctional components resulting in increased cytosolic levels of β-catenin and decreased levels of E-cadherin (18,23,24).

In this study, the underlying mechanisms and the functional effects of LMP1-mediated down-regulation of plakoglobin were analyzed. LMP1 decreased plakoglobin transcription but did not affect protein stability and these transcriptional effects did not require the activation of PI3K/Akt signaling. Both cytosolic and nuclear pools of plakoglobin were decreased by LMP1, however neither the loss nor the restoration of nuclear plakoglobin pools affected the association of Tcf4 with plakoglobin, nor did it correlate with Tcf/Lef activity. These findings indicate that to enhance migration, LMP1 primarily affects junctional proteins. This involves decreasing junctional plakoglobin and inducing N-cadherin to promote a switch from E-cadherin to N-cadherin, a feature associated with oncogenic transformation. β-catenin was also increased and stabilized by LMP1 in C666-1 cells, and although it had enhanced association with N-cadherin it did not significantly enhance Tcf/Lef association or promoter activity. Similarly, in NPC biopsy samples, plakoglobin levels were also reduced or not detectable. This study reveals that the effects of LMP1 on the adherens junctional proteins, plakoglobin and N-cadherin, likely contribute to the LMP1-induced migration and metastasis in NPC.

Materials and Methods

Tumor and normal tissue specimens

Normal gingiva were a kind gift from Dr. Jennifer Webster-Cyriaque (University of North Carolina). NPC specimens were described previously (25).

Cell culture

Culture and stable selection of C666-1 cell lines have been described previously (20).

Immunoprecipitation and immunoblot analysis

Cell lysates were prepared, quantified and analyzed by immunoblot as previously described (3,20). For immunoprecipitations, 200 μg of protein was used and performed as previously described (26). Densitometry was performed with Image J.

Cellular fractionations

Cytoplasmic and nuclear fractionations and, Triton-soluble fractions were prepared as previously described (8,27).

Antibodies and reagents

Rabbit anti-GAPDH (clone FL-335), goat anti-β actin (clone I-19), anti-GRP78 (clone N-20), anti-PARP (clone N-20) and mouse anti-ubiquitin (clone P4D1) were purchased from Santa Cruz. Mouse anti-γ-catenin, anti-β-catenin, anti-E-cadherin, anti-N-cadherin and anti-desmoglein 1 were purchased from BD Biosciences. Mouse anti-Tcf4 was purchased from Millipore. Rabbit polyclonal anti-γ-catenin for immunohistochemistry staining was purchased from Cell Signaling. The inhibitors LY294002 and Akt inhibitor I were purchased from Calbiochem.

Quantitative reverse transcriptase-PCR

Total RNA was prepared using the RNeasy PLUS Mini kit (QIAGEN). Primers for qRT-PCR are as follows: 5′CTGCTCGCCATCTTCAAGTC3′ and 5′ TGGTGATGGCATAGAACAGG3′ for plakoglobin; 5′ TCACCCACACTGTGCCCATCTACGA3′ and 5′ CAGCGGAACCGCTCATTGCCAATGG3′ for actin; 5′TGCACCACCAACTGCTTAGC3′ and 5′GCGGGGCCATCCACAGTCTT3′ for GAPDH. Quantitative RT-PCR was performed using QuantiTect SYBR green RT-PCR kit (Qiagen). Analysis and fold change in transcript levels was calculated as previously described (28).

Reporter assays

C666-1 cells were transfected with 900 ng pGL3-OT or pGL3-OF reporter plasmids, 100 ng pRL-TK (Promega), with or without 20 ng pLef-1 using Lipofectamine 2000 (Invitrogen). Luciferase assays were performed at 48 hr post transfection, using the Dual luciferase reporter assay (Promega). Background luciferase activity was measured using pGL3-OF containing three mutated Tcf4 sites. Luciferase activity was normalized to Renilla activity for transfection efficiency.

Immunohistochemistry and immunofluorescence

Immunohistochemistry staining was performed using anti-γ-catenin antibody (Cell Signaling) and detected with biotinylated anti-rabbit IgG (H+L) and streptavidin-alkaline phosphatase (Jackson ImmunoResearch) as previously described (19). For immunofluorescence, cells were fixed with 50% v/v methanol:acetone, blocked with 5% BSA, stained with primary antibody and detected with AlexaFluor 647 anti-rabbit IgG (Molecular Probes) for plakoglobin or FITC anti-mouse IgG (Sigma) for N-cadherin and β-catenin.

Results

LMP1 decreases plakoglobin transcription without affecting protein stability

LMP1 has been shown to down-regulate the expression of plakoglobin in the NPC cell line, C666-1 (20). To investigate the mechanism for this down-regulation of plakoglobin, LMP1 was stably expressed in C666-1 cells and evaluated for the loss of plakoglobin. Quantitative RT-PCR revealed that LMP1 induced a 50% reduction in levels of plakoglobin transcript (Fig. 1A). In the presence of the protein synthesis inhibitor, cyclohexamide, plakoglobin degraded at a similar rate in both pBabe control cells and LMP1-expressing cells (Fig. 1B). Plakoglobin has been described to be regulated by proteasome-mediated degradation (29,30). Treatment of the pBabe cells with the proteasome inhibitor MG132 increased the amount of plakoglobin over time however, the plakoglobin levels in LMP1-expressing cells remained constant (Fig.

1C). This difference is reflected in the 40% increase of plakoglobin in pBabe cells compared to LMP1-expressing cells (Fig. 1C). This protection of plakoglobin from degradation in LMP1-expressing cells may result from association with other proteins. Importantly, this also indicates that LMP1 does not down-regulate plakoglobin through enhanced proteasome-mediated degradation. Although these findings do not rule out the possible contributions of post-transcriptional modifications to the plakoglobin transcript by LMP1, collectively they do indicate that LMP1 affects plakoglobin transcription and not plakoglobin stability.

Activation of PI3K/Akt signaling is not required for down-regulation of plakoglobin

It has previously been shown that activation of PI3K/Akt and canonical NFκB signaling are required for LMP1-induced migration (20) however, constitutive activation of Akt signaling using a myristylated-Akt construct did not decrease plakoglobin levels (20). To further examine the requirement of PI3K/Akt signaling in LMP1-mediated down-regulation of plakoglobin, PI3K and Akt signaling were inhibited by treatment with LY294002 and Akt inhibitor I, respectively. The PI3K/Akt inhibitors were used at the same concentrations as those used to inhibit LMP1-induced migration. Quantitative RT-PCR revealed that inhibition of PI3K/Akt did not affect the decreased plakoglobin transcription (Fig. 2A). Plakoglobin protein levels were also not restored by the inhibitors (Fig. 2B). Although LY294002 was more potent than the Akt inhibitor I at reducing the phosphorylation and activation of Akt, both inhibitors were effective (Fig. 2B).

Although it has previously been shown, using cells stably expressing an IκBα super-repressor, that blocking canonical NFκB signaling prevented LMP1-mediated down-regulation of plakoglobin (20), this result has been variable. This may reflect varying efficiencies of the IκBα super-repressor or that several distinct NFκB components may contribute to the regulation of plakoglobin by LMP1.

Overall, these findings indicate that LMP1-decreased plakoglobin transcription is not mediated through activation of PI3K/Akt and that the involvement of canonical NFκB signaling if any, is minimal. As both PI3K/Akt and canonical NFκB pathways contribute to LMP1-induced migration, the effects are either distinct from the effects on plakoglobin or act downstream from LMP1's induced loss of plakoglobin.

LMP1 down-regulates both cytosolic and nuclear pools of plakoglobin

Plakoglobin functions primarily in three different sub-cellular locations: as a junctional protein on the plasma membrane, as a free cytosolic component that competes with β-catenin for the Axin degradation complex, and as a nuclear transcription factor in Tcf/Lef signaling. To determine the effects of LMP1 on cytosolic and nuclear pools of plakoglobin, cells were fractionated using GRP78 and PARP as respective markers. Of note, the cytosolic fraction would contain both plasma membrane and cytosolic components. Plakoglobin levels were reduced in both cytosolic and nuclear fractions of LMP1-expressing cells, and increased in both fractions of LMP1-expressing cells with restored plakoglobin (LMP1/PG) (Fig. 3A).

β-catenin shares common binding partners with plakoglobin including E-cadherin in adherens junctions, Axin in the cytosol, and Tcf/Lef co-activators in the nucleus. It was therefore of interest to determine whether the effects on plakoglobin affected β-catenin levels in cytosolic and nuclear fractions. LMP1 has previously been shown to stabilize and induce β-catenin signaling, and in C666-1 cells LMP1 induced cytosolic and nuclear accumulation of β-catenin (Fig. 3A) (23,24). However, restoration of plakoglobin in LMP1 expressing cells (LMP1/PG) did not affect the cytosolic or nuclear levels of β-catenin compared to LMP1 expressing cells (Fig. 3A). This indicates that the inhibition of LMP1-induced migration by restoration of plakoglobin is not mediated through changes in the abundance of cytosolic or nuclear β-catenin.

To evaluate how LMP1 stabilized β-catenin, qRT-PCR and immunoblot analyses of cyclohexamide and MG132-treated C666-1 cells were performed. Quantitative RT-PCR revealed that LMP1 increased β-catenin mRNA levels by 50% (Fig. 3B). In the presence of cyclohexamide, β-catenin levels decreased in the pBabe control cells but were not decreased in the LMP1-expressing cells (Fig. 3C). Treatment with MG132 increased β-catenin levels in both pBabe and LMP1 cells, but the increase was higher in pBabe cells suggesting that there is more proteasome-mediated degradation in pBabe cells than LMP1 cells. These findings suggest that LMP1 enhances β-catenin stability partly through inhibiting proteasome-mediated degradation.

Ubiquitination can regulate proteasome-mediated degradation, protein localization and signal transduction. In breast cancer cells, mono- and poly-ubiquitination of β-catenin by the ubiquitin-conjugating enzyme Rad6B, is associated with its stabilization (31). However ubiquitination of β-catenin has been described to be both stabilizing and de-stabilizing. To investigate whether LMP1 affects β-catenin ubiquitination, C666-1 cells were treated with MG132 and ubiquitinated β-catenin was detected by immunoblotting. Mono- and poly-ubiquitinated β-catenin was more strongly detected in LMP1-expressing cells (Fig. 3D), indicating that LMP1 enhances β-catenin ubiquitination in C666-1 cells. Although LMP1 has been shown to inhibit the ubiquitin ligase Siah to stabilize β-catenin in B lymphocytes (24), LMP1 may employ other mechanisms that promote ubiquitination in C666-1 cells, such as those involving Rad6B. These findings indicate that LMP1 up-regulates β-catenin by both inducing its transcription and enhancing its stability, possibly through ubiquitin-mediated stabilization.

LMP1 effects on plakoglobin do not affect Tcf/Lef activity

To investigate whether the changes in nuclear plakoglobin and β-catenin induced by LMP1 affect Tcf/Lef signaling, Tcf/Lef reporter activity and the interaction of Tcf4 with plakoglobin or β-catenin were analyzed. LMP1 induced an approximate 2-fold increase in Tcf/Lef activity in both LMP1-expressing cells and LMP1/PG cells, compared to pBabe control cells or pBabe cells with over-expressed plakoglobin (pBabe/PG), respectively (Fig. 4A). The same fold increase occurred in the presence or absence of exogenously supplied Lef-1. Expression of plakoglobin in pBabe/PG or LMP1/PG cells induced an approximate 0.7-fold increase in Tcf/Lef activity, but this was only observed in the absence of exogenously supplied Lef-1 (Fig. 4A). These findings support that plakoglobin can act as a weak Tcf/Lef transcription factor (32,33), and show that LMP1 can weakly induce Tcf/Lef activity. However the decrease in nuclear levels of plakoglobin induced by LMP1 do not correlate with the increase in Tcf/Lef activity, suggesting that LMP1 effects on plakoglobin do not function at the level of Tcf/Lef activity.

To understand whether the increase in Tcf/Lef activity in LMP1-expressing cells is due to alterations in the amount of plakoglobin or β-catenin bound to Tcf/Lef factors, cytosolic and nuclear fractions of C666-1 cells were immunoprecipitated for Tcf4 and immunoblotted for plakoglobin or β-catenin. Tcf4 levels were comparable in both cytosolic and nuclear fractions of pBabe, LMP1 and LMP1/PG cells (Fig. 4B). Tcf4 mainly localizes to the cytosol and is imported into the nucleus directly bound to plakoglobin or β-catenin. The amounts of plakoglobin or β-catenin bound to cytosolic or nuclear Tcf4 were similar in the presence of LMP1 or with over-expressed plakoglobin suggesting that the effects of LMP1 on the levels of plakoglobin or β-catenin do not affect Tcf4 association. These findings were confirmed in reciprocal immunoprecipitations for plakoglobin and β-catenin (Fig. 4B). Although the amounts of plakoglobin or β-catenin immunoprecipitated differed between cell lines, the amounts of associated Tcf4 remained unchanged. These findings indicate that LMP1-induced changes in nuclear plakoglobin and β-catenin levels do not affect Tcf/Lef translocation or

association. Interestingly, Tcf/Lef promoter activity did not correlate with migration as previously shown to be induced by LMP1 or inhibited by restoration of plakoglobin (20).

Down-regulation of cytosolic pools of plakoglobin by LMP1 perturbs junctional assembly

To investigate whether down-regulating the cytosolic pool of plakoglobin by LMP1 affects junctional assembly, C666-1 cells were analyzed for the amount of plakoglobin associated with junctional proteins. Cells were first fractionated by Triton X100-solubility to differentiate between proteins that are Triton insoluble and found assembled at junctions and, Triton soluble proteins that exist freely in the cytosol or within the plasma membrane. E-cadherin, N-cadherin, β-catenin and plakoglobin were used as markers for adherens junction proteins. Plakoglobin is also found in desmosomes, and the desmosomal cadherin, desmoglein-1 (Dsg1), was used as a desmosomal marker. Desmoglein-1 was not detected in the Triton-insoluble fraction, suggesting that C666-1 cells do not contain desmosomes (Fig. 5A). In addition, the desmosomal linker protein, desmoplakin, was not detected in the plasma membrane by immunofluorescence (data not shown), further supporting that desmosomes are not assembled in C666-1 cells. The adherens junction markers were detected in the Triton-insoluble fraction indicating that C666-1 cells do contain assembled adherens junctions (Fig. 5A). The amount of β-catenin in the Triton-insoluble fraction was not altered, indicating that β-catenin induction by LMP1 does not affect the amount within adherens junctions. The down-regulation of E-cadherin by LMP1 has been suggested to enhance migration (34). In support of this, E-cadherin was detectable at low levels in the Triton-insoluble fraction in pBabe control cells but could not be detected in LMP1-expressing cells. Over-expression of plakoglobin increased E-cadherin in the Triton-insoluble fraction of the pBabe/PG cells. Interestingly, E-cadherin remained undetectable in the LMP1/PG cells despite the fact that these cells have reduced levels of migration (20). In LMP1-expressing cells, N-cadherin was greatly induced in the Triton-soluble fraction. In the Triton-insoluble fraction, LMP1 slightly increased the levels of N-cadherin which was dramatically increased by over-expression of plakoglobin. The combined loss of E-cadherin and gain of N-cadherin is representative of the “cadherin switch” observed in cancer progression (35), and may contribute to LMP1-induced migration. The interaction of junctional plakoglobin with N-cadherin has been shown to suppress tumor growth (36). In the LMP1/PG cells, the increased amounts of N-cadherin and plakoglobin in the Triton-insoluble fraction suggest that plakoglobin may form adherens junctions with N-cadherin to decrease migration (Fig. 5A).

To evaluate whether there are indeed changes in the interactions between plakoglobin and β-catenin with E-cadherin and N-cadherin, C666-1 cells were evaluated for E-cadherin or N-cadherin associated plakoglobin and β-catenin. In the whole cell lysates, E-cadherin levels were decreased in LMP1-expressing cells with or without restored plakoglobin when compared to pBabe-expressing cells (Fig. 5B). Plakoglobin readily immunoprecipitated with E-cadherin in pBabe-expressing cells, but not in LMP1-expressing cells with or without restored plakoglobin (Fig. 5B). Additionally, although increased levels of β-catenin were detected in the whole cell lysates of LMP1-expressing cells compared to pBabe-expressing cells, the level of β-catenin immunoprecipitating with E-cadherin was decreased in LMP1-expressing cells with or without restored plakoglobin (Fig. 5B). This suggests that there is a loss of E-cadherin associated junctions in LMP1-expressing cells.

N-cadherin was greatly induced in cells that expressed LMP1 (Fig. 5B). In these LMP1-expressing cells, N-cadherin levels were unchanged upon restoration of plakoglobin, and N-cadherin was readily immunoprecipitated with plakoglobin and β-catenin (Fig. 5B). The amount of plakoglobin bound to N-cadherin was increased in LMP1-expressing cells and further increased in LMP1-expressing cells with restored plakoglobin (Fig. 5B). Additionally, the amount of β-catenin bound to N-cadherin was increased in LMP1-expressing cells (Fig. 5B). However, the level of β-catenin in the Triton-insoluble fraction was unchanged (Fig. 5A),

indicating that LMP1 does not affect the total levels of junctional β-catenin. The reduction of junctional plakoglobin in the Triton-insoluble fraction (Fig. 5A) and the loss of E-cadherin associated junctions in LMP1-expressing cells (Fig. 5B) suggest that LMP1 may promote migration through an overall loss of junctional plakoglobin and a switch from E- to N-cadherin. Additionally, since E-cadherin remains down-regulated in LMP1/PG cells, the enhanced assembly of plakoglobin with N-cadherin likely contribute to the reduced migration observed in LMP1/PG cells (20).

To evaluate the interaction between plakoglobin and N-cadherin at the plasma membrane, plakoglobin and N-cadherin were visualized by immunofluorescence. Plakoglobin was detected in pBabe and pBabe/PG cells at punctate points between cell-cell contacts, and in the cytosol of cells expressing high levels of plakoglobin in LMP1/PG cells (Fig. 6A). In LMP1-expressing cells, N-cadherin was detected at the plasma membrane and the merged image demonstrated co-localization with plasma membrane associated plakoglobin in LMP1/PG cells (Fig. 6A), further supporting that the enhanced association of plakoglobin with N-cadherin occurs at the junctional level. To identify the effect of LMP1-induced loss of junctional plakoglobin on other adherens junction proteins, immunofluorescence of β-catenin was performed. Although there was a slight increase in detection of cytosolic β-catenin in LMP1-containing cells, the majority of β-catenin remained localized to the plasma membrane, where at points of cell-cell contacts it co-localized with plakoglobin (Fig. 6B). This observation and the lack of change in β-catenin levels in the Triton-insoluble fraction (Fig. 5A) suggest that LMP1 does not enhance migration through regulating total levels of junctional β-catenin. Additionally, the merged image of LMP1/PG cells showed that in cells expressing high levels of plakoglobin, there was a loss in the detection of plasma membrane associated β-catenin and an increase in cytosolic β-catenin. This observation and the lack of change in the levels of N-cadherin bound β-catenin in LMP1/PG cells (Fig. 5B), suggest that relocalization of plasma membrane associated, but non-junctional β-catenin to the cytosol, may also contribute to the inhibition of LMP1-mediated migration by restoration of plakoglobin.

Plakoglobin is down-regulated in NPC biopsies

To assess the levels of plakoglobin in NPC, immunohistochemistry staining was performed on primary NPC biopsies. The NPC specimens tested included examples from all WHO types (1-differentiated keratinizing squamous cell carcinoma, 2-non-keratinizing carcinoma and 3-undifferentiated carcinoma). The NPC specimens were representative of southeastern Asia origin since the majority (12/19) of these NPC specimens had the prevalent China1 strain of LMP1 as determined by heteroduplex assays (data not shown). In normal gingiva specimens, plakoglobin was detected with strong intensity at the characteristic cell-cell borders (Fig. 6C). In contrast, weak cytosplasmic staining was detected in 8/19 NPC specimens and was not detected in 11/19 (Fig. 6C). The localization and intensity of plakoglobin stain did not differ among the WHO types. These observations indicate that the loss of junctional plakoglobin is a general characteristic of NPC.

Discussion

LMP1 has been well documented to have oncogenic properties that can transform various epithelial cell lines resulting in enhanced growth and migration (3,4,37). In the EBV positive C666-1 NPC cell line, LMP1 induced growth in soft agar and enhanced migration, requiring the activation of both PI3K/Akt and canonical NFκB signaling (20). These studies also indicated a causal link between plakoglobin loss and LMP1-induced migration (20). LMP1-induced down-regulation of plakoglobin has also been observed in Rat-1 fibroblasts and MDCK canine kidney epithelial cells (14,34). However, as presented here the inhibition of PI3K/Akt signaling did not impair the ability of LMP1 to down-regulate plakoglobin. The

effects of inhibiting canonical NFκB signaling on the regulation of plakoglobin by LMP1 were also not reproducibly apparent (20). The inhibition of LMP1-induced migration by PI3K/Akt and canonical NFκB inhibitors (20) likely result from effects in addition to the decrease in plakoglobin.

The induction of migration and metastasis has been linked to loss of both adherens junctions and desmosomes, (35,38). Plakoglobin is found in both types of junctions, but in plakoglobin null mice the assembly of desmosomal proteins is primarily affected (39). C666-1 cells apparently lack desmosomes as evidenced by the lack of Triton-insoluble Dsg1, plasma membrane localization of Dsg1, and the desmosomal linker protein desmoplakin. However, C666-1 cells do contain adherens junctions indicated by the presence of E-cadherin, β-catenin, and plakoglobin in the Triton-insoluble fraction (Fig. 5A), and the localization of β-catenin and plakoglobin to the plasma membrane (Fig. 6B). The expression of LMP1 in C666-1 cells decreased both E-cadherin and plakoglobin in the insoluble fraction indicating that LMP1 affects the components of the adherens junctions (Fig. 5).

The contribution of plakoglobin to cancer development has been linked to both adhesion-dependent and -independent mechanisms. Like its closely related Armadillo family member, β-catenin, plakoglobin can also act as a transcription factor in Tcf/Lef-mediated responses. In addition, other adherens junction components like E-cadherin and p120-catenin, have also been shown to have a dual role, and can affect transcription through inhibition of Kaiso-mediated repression of transcription (40). However, the ability of plakoglobin to regulate Tcf/Lef activity and its link to transformation is still contradictory. Re-introduction of plakoglobin into lung cancer cells that have lost plakoglobin, reduces Tcf/Lef reporter activity and inhibits growth (41). In addition, expression of a dominant negative Tcf4 can inhibit the growth induced by plakoglobin over-expression in rat kidney epithelial cells (32). The data presented here indicate that the LMP1 decreased the association between junctional plakoglobin with E-cadherin but did not affect the interaction of either β-catenin or plakoglobin with Tcf4 to significantly alter transcription (Fig. 4B).

LMP1 has been shown to induce epithelial-mesenchyme transition (EMT) in MDCK cells (42). Cancer progression and the induction of EMT require the disruption of both adherens junctions and desmosomes (43) and the absence of desmosomes in C666-1 cells suggests that these cells have some characteristics of metastatic cells. LMP1 can further advance this cancer phenotype by inducing a cadherin switch. Epithelial cells typically express E-cadherin in adherens junctions. The inappropriate expression of N-cadherin in epithelial cells is the key switch that initiates the loss of E-cadherin and the disruption of cell-cell adhesion (44). However, the loss of E-cadherin alone is insufficient at inducing invasion (45), suggesting that in addition to the loss of cell-cell contacts, enhanced migration also requires the induction of N-cadherin. LMP1 targets both E-cadherin and N-cadherin resulting not only in the disruption of E-cadherin associated junctions but also in the aberrant induction of N-cadherin associated junctions and possible signaling effects. Although enhancing the association of plakoglobin with N-cadherin may explain the decrease in migration observed in LMP1/PG cells, the loss of E-cadherin associated plakoglobin and β-catenin likely also contributes to LMP1-induced migration. In addition, LMP1 greatly induced the accumulation of cytosolic or plasma membrane associated levels of free β-catenin (Fig. 3A and Fig. 5A) that perhaps may also contribute to migration in ways independent of adhesion or Tcf/Lef-mediated transcription.

In summary, this study reveals that the ability of LMP1 to enhance migration reflects its changes in the junctional assembly of plakoglobin, E-cadherin, and N-cadherin rather than the potential effects on Tcf/Lef regulated transcription. The ability of over-expression of plakoglobin to impair this migration likely reflects the assembly of plakoglobin and N-cadherin in adherens junctions. Importantly, plakoglobin expression is also lost in NPC biopsies, and

this loss is characteristic of all WHO types of NPC and is thus independent of the differentiation state of the tumor (Fig. 6C). This observed loss of plakoglobin by LMP1 is also a characteristic of EBV-associated NPC that may contribute to its highly metastatic nature.

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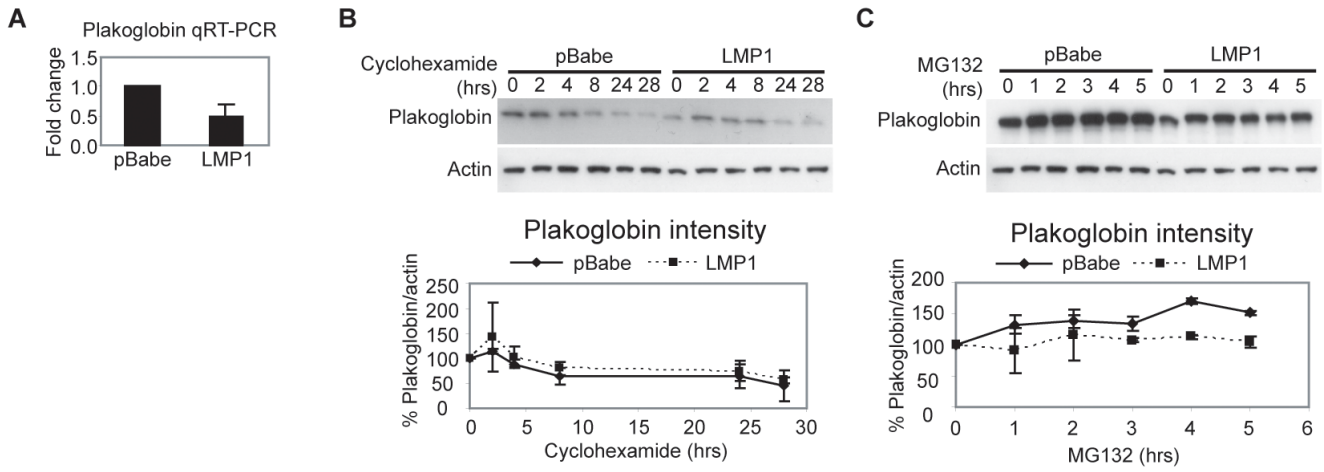

Figure 1.

LMP1 down-regulates plakoglobin transcription without affecting protein stability. (**A**) Quantitative RT-PCR for plakoglobin transcript in C666-1 cells. Fold change represents the change in plakoglobin transcript, normalized to actin, relative to pBabe and were averaged from 7 experiments. Error bars denote standard deviations. Immunoblot analysis showing plakoglobin levels upon treatment with (**B**) 20 μmol/L cyclohexamide and (**C**) 10 μmol/L MG132. Shown are representative results from duplicate drug treatments. Plakoglobin levels were normalized to actin and displayed relative to 0 hr in the graphs below. Error bars represent standard deviations from immunoblots of duplicate cyclohexamide and MG132 treatments.

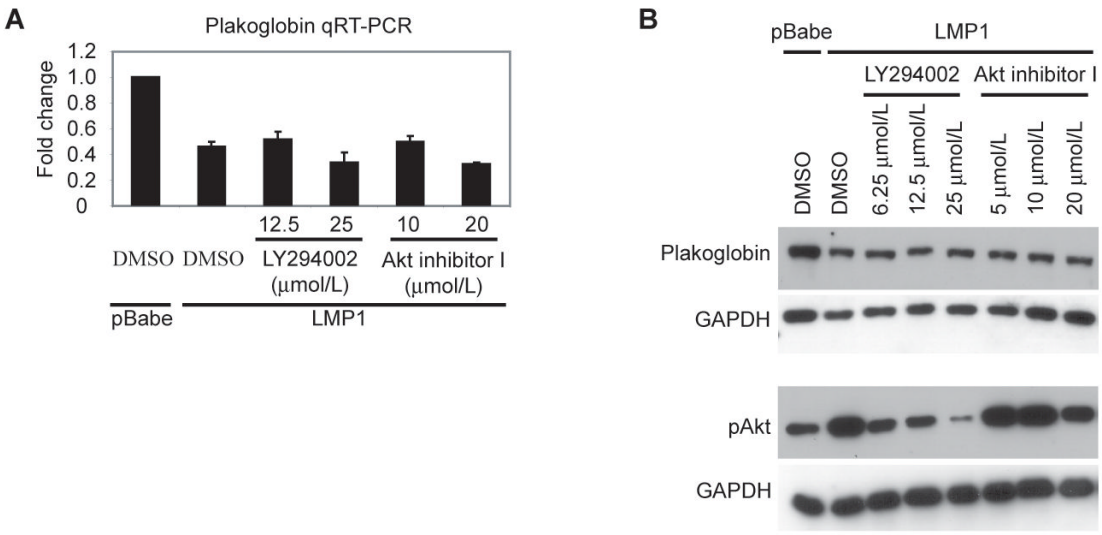

Figure 2.

PI3K/Akt signaling is not required for LMP1-mediated down-regulation of plakoglobin. (**A**) Quantitative RT-PCR for plakoglobin transcript in C666-1 cells upon overnight treatment with LY294002 or Akt inhibitor I. Fold change represents the change in plakoglobin transcript, normalized to GAPDH, relative to pBabe and averaged from 3 real-time measurements. Error bars denote standard deviations. (**B**) Immunoblot analysis of C666-1 cells upon overnight treatment with LY294002 or Akt inhibitor I. GAPDH was used as a loading control. Shown are representative blots from 3 immunoblots of duplicate drug treatments.

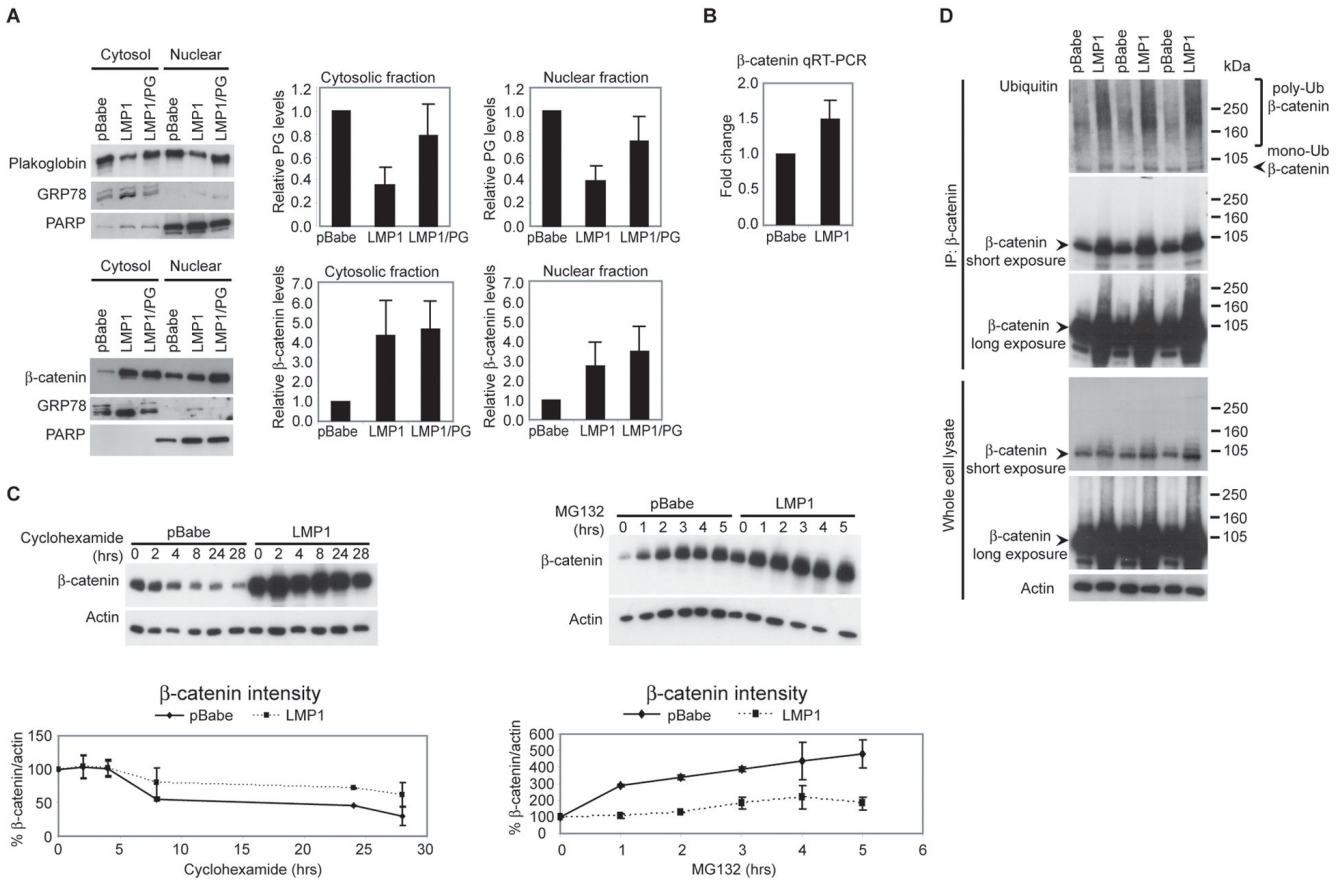

Figure 3.

LMP1 affects both cytosolic and nuclear pools of plakoglobin and β-catenin. (**A**) Immunoblot analysis of cytosolic and nuclear fractions from pBabe, LMP1 and LMP1/plakoglobin (LMP1/PG)-expressing C666-1 cells for plakoglobin and β-catenin levels. Shown are representative blots from triplicate fractionations. Plakoglobin and β-catenin levels from triplicate fractionations are displayed graphically. Error bars denote standard deviations. (**B**) Quantitative RT-PCR for β-catenin transcript in C666-1 cells. Fold change represents the change in β-catenin transcript, normalized to GAPDH, relative to pBabe and were averaged from 3 experiments. Error bars denote standard deviations. (**C**) Immunoblot analysis showing β-catenin levels upon treatment with 20 μmol/L cyclohexamide and 10 μmol/L MG132. Shown are representative results from duplicate drug treatments. β-catenin levels were normalized to actin and displayed relative to 0 hr in the graphs below. Error bars represent standard deviations from immunoblots of duplicate cyclohexamide and MG132 treatments. (**D**) Immunoblot analysis for ubiquitinated β-catenin from triplicate samples and immunoprecipitations for β-catenin from C666-1 cells. Cells were treated with 10 μmol/L MG132 for 2 hrs to facilitate the detection of ubiquitinated proteins.

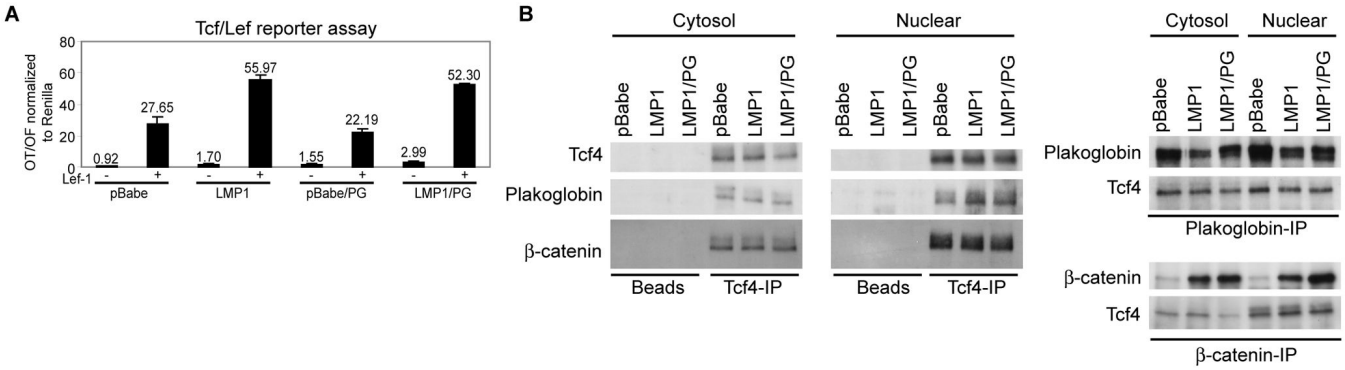

Figure 4.

Down-regulation of nuclear pools of plakoglobin by LMP1 does not affect Tcf/Lef activity. (**A**) Tcf/Lef reporter activity of C666-1 cells. Values represent the Tcf/Lef-specific luciferase activity after normalization for transfection efficiency to Renilla activity, from triplicate samples. Error bars denote standard deviations. (**B**) Immunoprecipitation for Tcf4 from cytosolic and nuclear fractions of pBabe, LMP1 and LMP1/plakoglobin (LMP1/PG)-expressing C666-1 cells, for Tcf4-associated plakoglobin and β-catenin. Reciprocal immunoprecipitations were performed to control for the immunoprecipitation. Beads alone were used as negative controls for non-specific binding. Shown are the results from one fractionation.

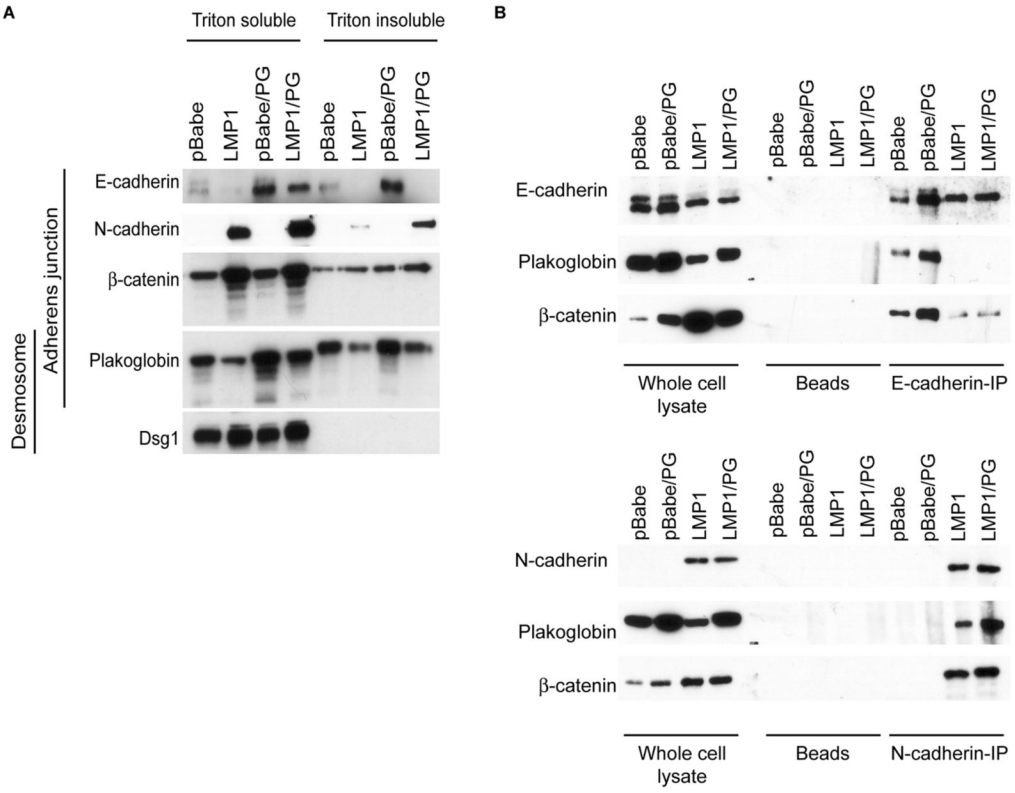

Figure 5.

Down-regulation of cytosolic plakoglobin by LMP1 pertubes junctional assembly. (**A**) Immunoblot analysis of triton-soluble and insoluble fractions from pBabe, pBabe/plakoglobin (pBabe/PG), LMP1 and LMP1/plakoglobin (LMP1/PG)-expressing C666-1 cells. Shown are the representative results from duplicate immunoblots. (**B**) Immunoprecipitation for N-cadherin associated plakoglobin and β-catenin in C666-1 cells. Beads alone were used as negative controls for non-specific binding. Shown are the representative results from immunoprecipitations of duplicate samples.

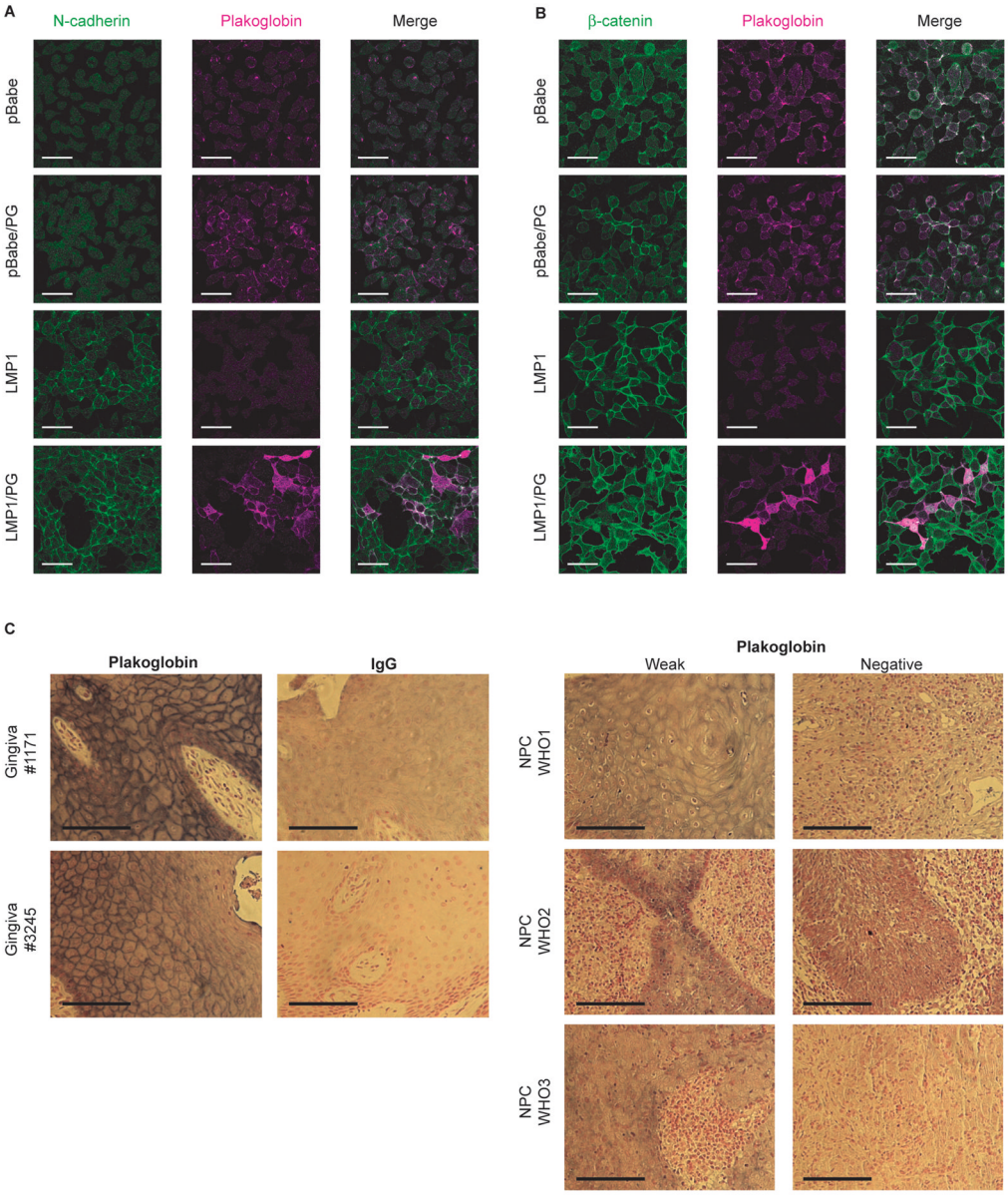

Figure 6.

Plakoglobin co-localizes with N-cadherin and its expression is lost in NPC. (**A, B**) Immunofluorescence of pBabe, pBabe/plakoglobin (pBabe/PG), LMP1 and LMP1/plakoglobin (LMP1/PG)-expressing C666-1 cells for N-cadherin, plakoglobin and β-catenin. N-cadherin and β-catenin staining are detected as green using the FITC fluorophore, plakoglobin staining is artificially colored purple and detected with AlexaFluor 647, the merge image was assembled using ImageJ software. Images were acquired at 400X-magnification in oil immersion. Scale bar, 50 μm. (**C**) Immunohistochemistry staining for plakoglobin in normal gingiva and NPC biopsies. Rabbit IgG was used as a negative control. Images were acquired at 200X-magnification using the Zeiss Axioskop microscope. Scale bar, 50 μm.